#11 01/26/99

Docket No.: 50356-150 (715-118) PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :

Nobuhiko KAYAGAKI, et al :

Serial No. 08/913,555 Group Art Unit: 1644

Filed: September 19, 1997 Examiner: M. Tung

For: MONOCLONAL ANTIBODY SPECIFICALLY REACTING WITH Fas LIGAND AND PRODUCTION PROCESS THEREOF

SUPPLEMENTAL RESPONSE

Honorable Commissioner of
Patents and Trademarks
Washington, D. C. 20231

Sir:

It appears from our records that the certified English translated priority document of Japanese Patent Application No. 87420/1995 was not attached to our Supplemental Response filed January 13, 1999. Attached herewith is the translated Japanese Patent Application to complete the filed Supplemental Response.

Respectfully submitted,

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DECLARATION

I, Shigeaki Nishikawa of Nishikawa & Associates, Visual City, Suite 401, 43-8, Higashi-Nippori 3-chome, Arakawa-ku, Tokyo, Japan, do solemnly and sincerly declare that I am well acquainted with both the Japanese language and the English language and that the attached English translation of an officially certified copy of Japanese Patent Application No. 87420/1995 is a true and correct translation to the best of my knowledge and belief from the Japanese language to the English language.

Dated this 25th day of November, 1998

Shigeaki Nishikawa

Shigeaki Nishikawa
(translator)

(Translation)

PATENT OFFICE

JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this Office.

Date of Application : March 20, 1995

Application Number : Patent Application No. 087420/1995

Applicant(s) : SUMITOMO ELECTIC INDUSTRIES, LTD.

Dated : April 12, 1996

Yuji Kiyokawa

Commissioner, Patent Office

Certificate No. Hei 08-3021132

[Document Name] Application for Patent

[Docket Number] 94YA0288

[Filing Date] March 20, 1995

[To] Hon. Director General of Patent Office

[Internationl Patent Classification]
C07K 15/23

[Title of the Invention] MONOCLONAL ANTIBODY SPECIFICALLY REACTING WITH Fas LIGAND AND PRODUCTION PROCESS THEREOF

[Number of Claims] 11

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[List of Materials Submitted]

[Material Name] Specification 1

[Material Name] Drawings 1

[Material Name] Abstract 1

[General Power of Attorney Number] 9003720

[Document Name] Specification

[Title of the Invention] MONOCLONAL ANTIBODY SPECIFICALLY REACTING WITH Fas LIGAND AND PRODUCTION PROCESS THEREOF

[Claims]

[Claim 1] A monoclonal antibody which specifically reacts with a Fas ligand, or an active fragment thereof.

[Claim 2] The monoclonal antibody or the active fragment thereof according to Claim 1, wherein a species of the Fas ligand is the human.

[Claim 3] The monoclonal antibody or the active fragment thereof according to Claim 1, wherein a species of the Fas ligand is a mouse.

[Claim 4] The monoclonal antibody or the active fragment thereof according to Claim 1, which is a mouse-derived monoclonal antibody.

[Claim 5] The monoclonal antibody or the active fragment thereof according to Claim 1, which can inhibit a physiological reaction between the Fas ligand and Fas.

[Claim 6] A process for producing monoclonal antibodies specifically reacting with a Fas ligand, which comprises the steps of (1) immunosensitizing an animal with a Fas ligand on which the Fas ligand has been expressed, (2) preparing antibody-producing cells from the immunosensitized animal to form a suspension of the antibody-producing cells, (3) mixing the suspension of the antibody-producing cells with myeloma cells to fuse both

cells, (4) diluting the fused cells with a medium which does not favor unfused myeloma cells to culture the fused cells, thereby sorting hybridomas produced by the fusion of the antibody-producing cells with the myeloma cells, (5) determining whether antibodies secreted in a culture supernatant containing the hybridomas are against the desired antigen or not using, as an indicator, the fact that the antibodies inhibit the attack of a Fas ligand present in a supernatant of Fas ligand-expressed COS cells against Fas-expressed cells, (6) cloning a series of cells in culture wells in which cells secreting the desired antibodies exist, (7) selecting a clone from which the desired antibody is secreted, (8) conducting cloning again to establish a hybridoma clone which secretes a monoclonal antibody against the desired antigen, and (9) preparing the monoclonal antibody from a culture supernatant of the hybridoma or ascites fluid obtained by intraperitoneally administering the hybridoma to a mouse.

[Claim 7] The production process according to Claim 6, wherein the animal is a rodent belonging to MRL lpr/lpr mice.

[Claim 8] The production process according to Claim 6, wherein the myeloma cell is P3X63Ag8.653.

[Claim 9] A hybridoma which produces a monoclonal antibody specifically reacting with a Fas ligand present on a cell surface.

[Claim 10] A method of detecting a Fas ligand in a

solution, which comprises combining a plurality of monoclonal antibodies against Fas ligand with each other.

[Claim 11] A kit for use in detecting a Fas ligand, comprising in combination a plurality of monoclonal antibodies against Fas ligand.

[Detailed Description of the Invention]

[0001]

[Industrial Field of Utilization]

The present invention relates to monoclonal antibodies which specifically react with a Fas ligand present on a cell surface, active fragments thereof, a method of detecting a Fas ligand, and kits for use in detecting a Fas ligand. The present invention also relates to a process for producing monoclonal antibodies which specifically react with a Fas ligand present on a cell surface, and hybridomas separately producing these monoclonal antibodies. The monoclonal antibodies according to the present invention are useful in elucidation of a Fas system and the like in cell death, immunothearpy and immunodiagnoses, detection of a Fas ligand, and industrial fields associated with them.

In the present invention, the active fragments mean fragments having the antigen-antibody reaction activity of the antibodies. Specific examples thereof include $F(ab')_2$, Fab', Fab, Fv and recombinant Fv.

[0002]

[Prior Art]

Multicellular organisms skillfully control the proliferation and death of cells to maintain their homeostasis. Many cells are removed by cell death in the course of ontogeny. In an adult, cells constituting organs always maintain their functions while keeping a balance between their proliferation and death. Such cell death is preliminarily programmed death called "programmed cell death" and is distinguished from "accidental cell death" caused by physical and chemical factors. These two deaths are different from each other in process. More specifically, the programmed cell death is caused by a process of apoptosis, while in the accidental cell death, cells are killed via a process of necrosis.

[0003]

A Fas antigen is a cell-surface protein that mediates cell death (apoptosis). Recently, a cDNA of the Fas antigen was cloned jointly by Dr. Naoto Ito, Dr. Shigekazu Nagata et al. in Osaka Bioscience Institute (Cell, Vol. 66, pp. 223-243, 1991). It was found from the structure of the cDNA thus obtained that a human Fas antigen is a transmembrane protein consisting of 319 amino acid residues and has one transmembrane region. The extracellular region of the Fas antigen is constituted by 157 amino acid residues and has a cysteine residue-rich structure. A mouse Fas antigen consists of 306 amino acid residues and has a homology of 49.3% with the human Fas antigen.

[0004]

It was found that the cysteine residue-rich structure of the extracellular region in the Fas antigen is a well conserved structure recognized in a low-affinity receptor of NGF (nerve growth factor) and a receptor of TNF (tumor necrosis factor). This fact revealed that the Fas antigen is a cell-surface protein belonging to the NGF/TNF receptor family. Since many of proteins belonging to this family have their ligands in the vital body, the Fas antigen is also expected to have its ligand in the vital body. A molecule of a rat Fas ligand was identified by a group of Dr. Shigekazu Nagata et al. in Osaka Bioscience Institute in 1993 (Cell, Vol. 75, pp. 1169-1178, 1993), and subsequently molecules of mouse and human Fas ligands were identified by the same group (Int. Immunol., Vol. 6 No. 10, pp. 1567-1574).

[0005]

It has been understood that the Fas antigen mediates a signal of "death" to cells. Besides, an anti-Fas antibody induces apoptosis against certain cells. In a mouse having lpr (lymphoproliferation) mutation exhibiting the symptom of autoimmune disease, it has been found that the mutation exists in its Fas gene. These results suggest that the inactivation of proteins mediating apoptosis, such as the Fas antigen, causes abnormal proliferation of cells, while abnormal activation thereof causes certain inflammatory reactions.

[0006]

As described above, the researches of Fas antigens prove that in an immune system, a system mediating a signal of "death" works from the outside of cells. However, there has been yet no knowing whether the cell death in development and neurocytes is induced by a like signal from the outside (the system of Fas works) or programmed in cells as called programmed cell death. Its elucidation is an important problem in future.

[0007]

A signal transfer mechanism for inducing apoptosis against cells, i.e., a problem that apoptosis is induced from a Fas antigen by what signal transfer mechanism, is also not elucidated. In order to exactly understand the system of Fas, it is necessary to make a ligand of the Fas (Fas ligand) and its function clear and to reconsider the system of Fas from the viewpoint of the interaction between ligand and receptor.

As described above, the gene of a Fas ligand was identified by Dr. Shigekazu Nagata et al. As a result, according to the above literature, "Cell", it has been found that the Fas ligand is a protein consisting of 278 amino acids with a molecular weight of 31,138, and it has also been found that 4 N-glycoside-bond sites exist therein, and it is hence a glycoprotein (Cell Technology, Vol. 13 No. 8, pp. 738-744, 1994).

[0008]

The report in literature by Hanabuchi et al. (Proc. Natl. Acad. Sci. USA, Vol. 91, No. 11, pp. 4930-4934, 1994) has showed that as a result of the analysis of the mechanism of lysing target cells by killer T cells via a Fas antigen, there is a possibility that the transmission of an apoptosis signal via the Fas antigen on the target cells may take part in the lysis of the target cells by $CD4^+$ T cells (CTL) which do not express perforin. This has revealed that a Fas ligand exists on the cell surface of $CD4^+$ CTL.

In a mouse having gld (generalized lymphoproliferative disease) mutation exhibiting the symptom of autoimmune disease, it has been found that the mutation exists in its Fas gene (Cell, Vol. 76, pp. 969-979, 1994).

[0009]

However, the recognition that a Fas ligand may play an important role in vital reactions has been just gained under circumstances. As described above, the Fas ligand molecule has been just identified at present, and so the mechanism of Fas and the Fas ligand has been just started to be elucidated. In order to make this mechanism clear, analysis at the protein level (immunological analysis), or acquisition of neutralizing antibodies or the like which inhibit the binding action of Fas to the Fas ligand is essential.

[0010]

[Problems Sought for Solution by the Invention]

It is an object of the present invention to provide monoclonal antibodies, which specifically react with a Fas ligand present on a cell surface, active fragments thereof, a production process of the monoclonal antibodies, and hybridomas separately producing the monoclonal antibodies.

Another object of the present invention is to provide monoclonal antibodies which can inhibit a physiological reaction between a Fas ligand and Fas, and specifically react with the Fas ligand.

[0011]

A still further object of the present invention is to detect a Fas ligand in a solution and a kit for use in detecting the Fas ligand.

The present inventors have considered that when a monoclonal antibody against Fas ligand is produced, the analysis of a Fas system will be advanced, and carried out an extensive investigation. As a result, the inventors have succeeded in acquiring monoclonal antibodies which specifically react with a Fas ligand, and hybridomas separately producing such antibodies.

The present inventors have further continued researches on the antibodies specifically reacting with a Fas ligand, and the like, and the present invention has been led to completion on the basis of the results of the researches.

[0012]

[Means for the Solution of the Problems]

According to the present invention, there are provided monoclonal antibodies, which specifically react with a Fas ligand, or active fragments thereof.

According to the present invention, there is still further provided a process for producing monoclonal antibodies specifically reacting with a Fas ligand, which comprises the steps of (1) immunosensitizing an animal with a Fas ligand cells on which the Fas ligand has been expressed, (2) preparing antibody-producing cells from the immunosensitized animal to form a suspension of the antibody-producing cells, (3) mixing the suspension of the antibody-producing cells with myeloma cells to fuse both cells, (4) diluting the fused cells with a medium which does not favor unfused myeloma cells to culture the fused cells, thereby sorting hybridomas produced by the fusion of the antibody-producing cells with the myeloma cells, (5) determining whether antibodies secreted in a culture supernatant containing the hybridomas are against the desired antigen or not using, as an indicator, the fact that the antibodies inhibit the attack of a Fas ligand present in a supernatant of Fas ligand-expressed COS cells against Fas-expressed cells, (6) cloning a series of cells in culture wells in which cells secreting the desired antibodies exist, (7) selecting a clone from which the desired antibody is secreted, (8) conducting cloning again to establish a hybridoma clone which secretes a monoclonal

antibody against the desired antigen, and (9) preparing the monoclonal antibody from a culture supernatant of the hybridoma or ascites fluid obtained by intraperitoneally administering the hybridoma to a mouse.

[0013]

According to the present invention, there are yet still further provided hybridomas separately producing monoclonal antibodies which specifically react with a Fas ligand present on a cell surface, a method of detecting a Fas ligand in a solution, which comprises combining a plurality of monoclonal antibodies against Fas ligand with each other, and a kit for use in detecting a Fas ligand, comprising a plurality of monoclonal antibodies against Fas ligand in combination.

[0014]

The present invention will hereinafter be described in detain.

A Fas ligand is a ligand of a Fas antigen that is a cell-surface protein mediating apoptosis. The identification of its gene has revealed that the Fas ligand is a protein consisting of 278 amino acids with a molecular weight of 31,138. Human, rat and mouse Fas ligands have been identified up to the present. The present invention is generally intended for the Fas ligands. Of these, the Fas ligands, the species of which are the human and mouse, are particularly preferred. Namely, the present invention relates to monoclonal

antibodies which specifically react with the respective ligands of human and mouse Fas antigens, and active fragments thereof.

[0015]

No particular limitation is imposed on the monoclonal antibodies according to the present invention so far as they specifically react with a Fas ligand. However, they can preferably inhibit a physiological reaction between a Fas ligand and Fas. The antibody, which inhibits the physiological reaction, as used herein means an antibody (neutralizing antibody) which can specifically bind to a binding site of a Fas ligand binding to Fas to prevent the Fas ligand from binding to Fas when a Fas ligand-expressed cell or a solubilized Fas ligand (sFas ligand) binds to a Fas-expressed cell to give a signal to the effect that the Fas-expressed cell is killed by apoptosis. Namely, when the monoclonal antibody which inhibits the physiological reaction of the Fas ligand with Fas is present, the Fas ligand-expressed cell fails to kill the Fas-expressed cell.

[0016]

Examples of the monoclonal antibodies according to the present invention, which specifically react with a Fas ligand, include respective monoclonal antibodies (NOK1 to NOK5) produced by hybridoma cell lines deposited as Accession Nos. FERM BP-5044 (Hybridoma NOK1), FERM BP-5045 (Hybridoma NOK2), FERM BP-5046 (Hybridoma NOK3), FERM BP-

5047 (Hybridoma NOK4) and FERM BP-5048 (Hybridoma NOK5) in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology.

Examples of other monoclonal antibodies according to the present invention, which specifically react with a Fas ligand, include antibodies the classes or subclasses of which are mouse IgG_1, mouse IgG_{2a}, mouse IgM and mouse IgG_3, respectively.

[0017]

The antibodies according to the present invention are useful not only for immunochemical researches, but also for immunotherapy, immunodiagnoses and the like. In order to achieve such objects, it is not always necessary to use the whole antibody molecule. A part of the molecule may be used so far as it is active. As easily understood by those skilled in the art, in some cases, it may be more preferable to use such a part of the molecule. Accordingly, the present invention also includes active fragments of the anti-Fas ligand antibodies. An antibody is a homogeneous immunoglobulin which recognizes a specific antigenic substance. The term "active fragment" means a fragment of an antibody active in antigen-antibody reaction. As specific examples thereof, may be mentioned $F(ab')_2$, Fab', Fab, Fv and recombinant Fv.

[0018]

The $F(ab')_2$ fragment is one of fragments obtained by digesting an immunoglobulin IgG with pepsin. When IgG is

subjected to pepsin digestion at a pH near 4.0, it is cleaved at a hinge area of its H chain to produce a fragment having a molecular weight of about 100,000. This cleavage takes place on the C-terminal side away from the disulfide bond between H chains. This fragment has two antigen-binding sites and hence can bind to antigens, thereby undergoing precipitin reaction and agglutination reaction. The Fab' fragment is a fragment produced by reducing the $F(ab')_2$ fragment with a reagent such as 2-mercaptoethanol and alkylating the reduced product with monoiodoacetic acid, thereby cleaving a disulfide bond between H chains, and having a molecular weight of about 50,000.

[0019]

The Fab fragment (antigen-binding fragment) is one of fragments obtained by the papain digestion of IgG. When IgG is subjected to papain digestion in the presence of cysteine, its H chain is cleaved at a site on the N-terminal side away from the disulfide bond between H chains in a hinge area, thereby producing two Fab fragments and one Fc fragment (crystallizable fragment). The Fab fragment is a fragment in which an Fd fragment (V_H domain + C_H1 domain) corresponding to about a half of the H chain on the N-terminal side is coupled to an L chain by a disulfide bond, said fragment having a molecular weight of about 45,000. The Fab fragment has one antigen-binding site. The Fv fragment is an antigen-binding fragment

composed of a variable region of immunoglobulin heavy chain (V_H) and a variable region of immunoglobulin light chain (V_L), said variable regions being coupled to each other by a nonconjugate bond.

[0020]

The recombinant Fv fragment can be obtained by sequencing a DNA from a hybridoma which produces a monoclonal antibody to determine base sequences which encode V_H and L_H, respectively, and then integrating these DNA fragments in a vector to produce a monovalent active antibody fragment having a structure of V_L-Linker-V_H. In IgG, Fab or F(ab')$_2$, V_H and L_H are coupled to each other by an S-S bond. In the recombinant Fv fragment, a linker is inserted between V_H and L_H so as to take the same configuration as the state coupled by the S-S bond. This fragment may be simply called "Fv" in some cases. It may also be called "scFv (single chain FV)". The recombinant Fv fragment may also be expressed by microorganisms such as *Escherichia coli* and bacteriophages.

[0021]

Although these fragments may be used singly, they may be bound to a substance such as albumin or polyethylene glycol to use them in the form of new complexes. In general, such a complex often exhibits its effect up to the maximum without being decomposed for a long period of time *in vivo*. A method of adding the substance such as albumin or polyethylene glycol to the

active fragment is described in, for example, Antibodies, A. Laboratory Manual, Cold Spring Harbor Laboratory, 1988. In general, the use of a divalent reaction reagent such as SPDP (product of Pharmacia) permits easily binding the active fragment to albumin or the like.

Humanized antibodies may also be provided by such methods as, for example, a mouse-derived active fragment is used to replace a primary structure other than complementarity-determing regions in both H chain and L chain by its corresponding primary structure in a human antibody.

[0022]

The monoclonal antibodies according to the present invention, and the hybridomas separately producing these monoclonal antibodies can be produced in accordance with the following process.

(1) An animal (for example, a rodent such as a mouse) is immunosensitized with COS cells which have expressed a Fas ligand.

(2) Antibody-producing cells are prepared from the immunosensitized animal to form a suspension thereof. Splenocytes or lymphadenocytes are mainly used. However, peripheral lymphocytes may also be used. When splenocytes are used, the spleen is taken out of the immunosensitized rodent to form a suspension of splenocytes.

[0023]

(3) The suspension of the antibody-producing cells

is mixed with myeloma cells to fuse both cells. For example, the suspension of the splenocytes is mixed with myeloma cells of a mouse in the presence of a hybridization accelerator (for example, polyethylene glycol) to fuse both cells. The cell fusion may be conducted by an electrical treatment. As the myeloma cells used herein, those (for example, 8-azaguanine-resistant strain) distinguishable from the antibody-producing cells in a subsequent selective culture are used.

(4) The fused cells are diluted with a medium which does not favor unfused myeloma cells to culture the fused cells, thereby sorting hybridomas produced by the fusion of the antibody-producing cell with the myeloma cell. More specifically, the fused cells are cultured in a selective medium in which the antibody-producing cells are viable, but the myeloma cells are killed, thereby sorting hybridomas produced by the fusion of the antibody-producing cell with the myeloma cell. For example, when 8-azaguanine-resistant myeloma cells are used, an HAT medium (hypoxanthine-aminopterine-thymidine containing medium) is used.

[0024]

(5) Whether antibodies secreted in a culture supernatant containing the hybridomas are against the desired antigen or not is determined using, as an indicator, the fact that the antibodies inhibit the attack of a Fas ligand present in a supernatant of Fas ligand-

expressed cells (for example, COS cells) against Fas-expressed cells.

(6) A series of cells in culture wells in which cells secreting the desired antibodies exist is cloned. The cloning is generally performed by the limiting dilution technique.

(7) A clone from which the desired antibody is secreted is selected.

(8) Cloning is conducted again to establish a hybridoma clone which secretes a monoclonal antibody against the desired antigen.

(9) A monoclonal antibody is prepared from a culture supernatant of the hybridoma or ascites fluid obtained by intraperitoneally administering the hybridoma to a mouse (for example, a nude mouse).

[0025]

More specifically, the monoclonal antibodies according to the present invention, and the hybridomas separately producing these monoclonal antibodies can be produced in accordance with the following process.

(1) Preparation of Fas ligand-expressed COS cells:

The gene of a human Fas ligand can be obtained by reference to the sequence described in S. Nagata et al., Int. Immunol. Vol. 6, No. 10, pp. 1567-1574. More specifically, respective complementary DNA primers as to both 5'-terminal and 3'-terminal sides of the Fas ligand cDNA were synthesized. Based on these primers, an

amplification reaction was conducted in accordance with the PCR technique using, as a template, a cDNA prepared from human killer T cells and containing a Fas ligand, and the resultant cDNA was then transfected into a vector, PMKitNeo. This Fas ligand gene-transfected vector was transfected into COS cells (ATCC CRL 1650) in accordance with the DEAE-dextran method to prepare human Fas ligand-expressed COS cells.

[0026]

(2) Immunosensitization:

A rodent (for example, MPL lpr/lpr mouse) is immunosensitized with the Fas ligand-expressed COS cells as an antigen. The reason why MPL lpr/lpr is used is that rodents including mice are observed expressing Fas in many tissues. Therefore, when a rodent such as a mouse is immunosensitized using, as an immunogen (= an antigen), Fas ligand-expressed cells, a signal of death mediated by Fas is inserted, resulting in killing the individual animal. It is therefore inconvenient to use such a rodent. As apparent from the report by Dr. Nagata et al. (Nature, Vol. 356, pp. 314-317, 1992), MPL lpr/lpr does not express a functional Fas. Therefore, if the MPL lpr/lpr mouse is inoculated with Fas ligand-expressed cells, the mouse is not killed, and so sufficient immunosensitization is feasible.

[0027]

(3) The spleen is taken out of the immunosensitized

rodent to form a suspension of splenocytes.

(4) The splenocytes of the immunosensitized mouse are mixed with myeloma cells of a mouse in the presence of a hybridization accelerator (for example, polyethylene glycol) to fuse both cells. As the myeloma cells, those (for example, 8-azaguanine-resistant strain) distinguishable from the antibody-producing cells in a subsequent selective culture are used.

(5) The fused cells are diluted with a medium which does not favor unfused myeloma cells to culture the fused cells, thereby sorting hybridomas produced by the fusion of the antibody-producing cell with the myeloma cell. More specifically, the fused cells are cultured in a selective medium (for example, an HAT medium) in which the antibody-producing cells are viable, but the myeloma cells are killed, thereby selectively culturing hybridomas produced by the fusion of the cell producing the intended antibody with the myeloma cell.

[0028]

(6) The presence of an antibody in a supernatant in each of culture wells separately containing the hybridomas is confirmed using, as an indicator, the fact that the antibodies inhibit the attack of a Fas ligand present in a supernatant of Fas ligand-expressed COS cells against Fas-expressed cells, namely, that killer activity is blocked. More specifically, there is a method in which a supernatant in each of culture wells separately containing

hybridomas is first reacted with the Fas ligand, and a transfectant which expresses a Fas antigen on a cell surface is then used as a target to determine whether the killer activity of the Fas ligand is blocked or not, thereby sorting hybridomas in culture supernatants which have blocked the killer activity.

(7) After the hybridomas which separately produce the desired antibody are selected, they are monocloned by the limiting dilution technique.

(8) A monoclonal antibody is collected from a culture supernatant of the monoclone.

[0029]

Since the monoclonal antibodies according to the present invention specifically react with a Fas ligand, they can serve to elucidate signal transfer mechanism for inducing apoptosis against cells, and a Fas system. In addition, the monoclonal antibodies according to the present invention and the active fragments thereof are useful in immunothearpy and immunodiagnoses, and industrial fields associated with them. For example, the monoclonal antibody specifically reacting with a Fas ligand is reacted with cells in blood, and a secondary antibody of a fluorescent marker is further bound thereto to measure the conjugate by flow cytometry or a fluorescent microscope, thereby being able to confirm that the Fas ligand has expressed in what cells. The binding of the monoclonal antibody according to the present invention

to a fluorochrome such as FITC or PE can be easily performed in accordance with a method known *per se* in the art. Accordingly, the monoclonal antibodies according to the present invention and the active fragments thereof are useful as reagents for diagnoses.

[0030]

When the monoclonal antibody according to the present invention is reacted with tissues and the like taken out of a patient suffered from various diseases (for example, an autoimmune disease, rheumatism and hepatitis), what tissue Fas ligand-expressed cells exist in can be determined.

A Fas ligand in a solution (blood, culture supernatant, body fluids, urine or the like) can be detected (further quantified) by using a plurality (for example, two kinds) of the monoclonal antibodies according to the present invention in combination. In addition, a kit for use in detecting a Fas ligand can be provided by using in combination a plurality (for example, two kinds) of the monoclonal antibodies against Fas ligand.

[0031]

[Examples]

The present invention will hereinafter be described more specifically by the following Examples. However, the present invention is not limited to these examples only.

[Example 1] <u>Preparation and characterization of monoclonal antibodies</u>

(1) Isolation of Fas ligand gene

① Preparation of primers

A human Fas ligand gene was isolated on the basis of the report by Nagata et al. More specifically, Xho I-5' FasL obtained by adding a sequence of 18mers of the 5' end of a human Fas ligand to a sequence of the Xho-I site on the 5' end side of human Fas ligand cDNA, and Not1-3' FasL obtained by adding a sequence of 18mers of the 3' end of a human Fas ligand to a sequence of the Not1 site on the 3' end side of human Fas ligand cDNA were separately subjected to DNA synthesis using Model 392 DNA/RNA synthesizer (manufactured by ABI) on a scale of 0.2 μmol. The product DNAs were purified in accordance with the protocol to prepare primers for PCR.

[0032]

② Preparation of template of Fas ligand cDNA

A template was prepared from human killer T cells in which a human Fas ligand had been expressed. More specifically, human killer T cells were activated with PMA and ionomycin to collect 1 x 10^7 cells. The collected cells were suspended in 1 ml of RNAzolB (product of Cosmo Bio). After 100 μl of chloroform were further added to the suspension, the mixture was left to stand for 30 minutes on an ice bath. Thereafter, a phenol layer was separated from a water layer by centrifugation (at 4°C) for 15 minutes at 15,000 rpm to recover only the upper water layer. An equiamount of isopropanol was added to the water

layer, and the resultant mixture was left to stand for 30 minutes at -80°C, followed by precipitation of RNA by centrifugation (15,000 rpm, 15 minutes, 4°C). After the precipitate thus obtained was centrifugally washed once with 1 ml of ethanol, it was suspended in 11.5 μl of water subjected to DEPC treatment. Added to this RNA suspension were 0.5 μl (0.5 mg/ml) of synthetic oligo dT, followed by a heat treatment for 10 minutes at 70°C. The mixture thus treated was then treated on an ice bath for 5 minutes.

[0033]

Thereafter, 4 μl of 5 x RT buffer (product of Stratagene), 1 μm of 10 mM dNTP, 2 μl of 0.1 M DTT and 1 μl of Superscript RTase (product of Stratagene) were added to conduct a reaction at 42°C for 50 minutes, thereby reversely transcribing RNA into cDNA. After the reaction mixture was treated at 90°C for 5 minute to deactivate the RTase, it was left to stand for 5 minutes on an ice bath. After 1 μl of RNaseH (product of Stratagene) was then added to this sample to conduct a reaction further for 20 minutes at 37°C, thereby decomposing unnecessary RNA to provide a template for cDNA containing Fas ligand.

[0034]

③ PCR

PCR was performed by reference to PCR Experimental Manual (HBJ Press, pp. 75-85) under the following conditions.

Namely, 1 μl of 10 mM dNTPmix (product of Pharmacia),

1 μl of Xho I Site-5' human FasL of 18mers (50 μM), 1 μl of Not I-3' human FasL of 18mers (50 μM), 4 μl of 10 x PCR buffer (product of Perkin-Elmer), 0.5 μl of Amplitaq TM (product of Perkin-Elmer) and 30.5 μl of water were added to 2 μl of the cDNA produced in Step ② into a solution of 40 μl in total. After this solution was topped with 40 μl of mineral oil (product of Sigma), an amplification reaction was carried out by means of a DNA thermal cycler for PCR (manufactured by Perkin-Elmer Japan). More specifically, the amplification reaction was carried out under conditions of successively 5 minutes at 94°C, 2 minutes at 55°C, 3 minutes at 72°C, 1 minute at 94°C, 2 minutes at 55°C and 10 minutes at 72°C by repeating the treatment between 2 minutes at 55°C and 1 minute at 94°C 30 cycles.

[0035]

④ Integration into PMKitNeo vector

After conducting the amplification reaction by PCR, only a water layer was extracted with a mixture of phenol and chloroform. Each 1.0 unit of Xho I and Not I (both, products of Boehringer Co.) were added to the extract thus obtained, and an accessory buffer was added, followed by a reaction at 37°C for 16 hours. The reaction mixture was electrophoresed in a 1% agarose gel. A band of about 850 bp corresponding to the Fas ligand was got out of the gel under UV irradiation.

DNA was extracted from this agarose gel using a

GENECLEAN II kit (product of BIO101, Funakoshi). More specifically, an accessory NaI solution was added to the gel to incubate the gel at 65°C for 10 minutes, thereby dissolving the gel in the solution. Glass milk was then added to the solution, and the mixture was rotationally stirred for 5 minutes to adsorb DNA on the glass milk. After this glass milk was washed three times with New-WASH solution, it was suspended in 10 μl of a TE buffer. The suspension was incubated at 65°C for 3 minutes, thereby dissolving DNA out of the glass milk.

[0036]

A PMKitNeo vector in an amount of 1 μg was then treated with the restriction enzymes Xho I and Not I in the same manner as described above to electrophorese it in a 0.75% agarose gel, followed by purification with the GENECLEAN II kit.

The Fas ligand cDNA and PMKitNeo vector were then ligated. More specifically, they were mixed so as to give a molar ratio of the vector to cDNA of 1:2, and the mixture was subjected to a ligation reaction at 16°C for 16 hours using a DNA ligation kit produced by Takara Shuzo Co., Ltd.

[0037]

⑤ Integration into *Escherichia coli*

The reaction mixture obtained in the step ④ was mixed with *Escherichia coli* competent cells (product of Toyobo) to incubate the mixture for 30 minutes on an ice

bath and for 40 seconds at 42°C, thereby inserting DNA into *Escherichia coli*. After an SOC medium was added thereto to conduct shaking culture at 37°C for 1 hour, the culture was poured into an LB agar medium containing ampicillin to conduct culture at 37°C for 1 day. Thereafter, appeared colonies were cultured at 37°C for 1 day in the LB medium, and the resultant plasmid (human Fas ligand-PMKitNeo) was then recovered by the alkali method.

[0038]

(2) Transfection into COS cell

The transfection of the plasmid (human Fas ligand-PMKitNeo) into COS cells (ATCC CRL 1650) was carried out in accordance with the DEAE-dextran method (Extra Issue of Experimental Medicine, Biomanual Series 4, Gene Transfection and Analysis of Expression, pp. 16-22, 1994, Yodo-sha). More specifically, DEAE-dextran produced by Armacia was used to perform the DEAE-dextran method in a proportion of (the human Fas ligand-PMKitNeo 5 μg)/(2 x 10^6 COS cells), thereby obtaining Fas ligand-expressed COS cells.

[0039]

(3) Immunosensitization

A suspension of the Fas ligand-expressed COS cells prepared in the step (2) was intraperitoneally injected into a Balb/c mouse in a proportion of 1 x 10^7 cells/mouse. After a week, the suspension of the Fas ligand-expressed COS cells was injected in the same mouse once a week, 3

times in total, thereby immunosensitizing the mouse.

[0040]

(4) Cell fusion

After 3 days from the final immunization, the spleen was taken out of the mouse. The spleen was minced, filtered through a mesh and then suspended in an RPMI 1640 medium (product of Nissui), thereby obtaining 1 x 10^8 splenocytes. The splenocytes and a mouse-derived 8-azaguanine-resistant strain (hypoxanthine-guanine phosphoribosyl transferase defective strain) P3X63Ag8.653 (ATCC CRL 1580) (1 x 10^7 cells) were mixed with each other in a proportion of about 5:1, and the resulting mixture was centrifuged (1500 rpm, 5 minutes).

[0041]

To the cell pellet thus obtained, 2 ml of a 50% solution of polyethylene glycol 4000 (product of Merck) in an RPMI 1640 medium were added over 1 minute with stirring on a hot water bath of 37°C. Added to the resulting mixture were 15 ml of an RPMI 1640 medium over 6 minutes with stirring, thereby conducting cell fusion. After the cell fusion, a great amount (about 40 ml) of an RPMI 1640 medium was added, and the mixture was centrifuged (1500 rpm, 5 minutes) to remove a supernatant. The splenocytes were then adjusted to 1 x 10^6 cells/ml with a 10% FCS (fetal calf serum)-RPMI 1640 medium (HAT medium) containing hypoxanthine (100 μM), aminopterine (0.4 μM) and thymidine (10 μM).

[0042]

(5) Selection of hybridoma

The cell suspension prepared in the step (4) was poured in 200-μl portions into 10 microplates each having 96 wells to culture the cells in a CO_2-incubator controlled at 37°C and CO_2 concentration of 5%. After a week, it was confirmed that only hybridomas formed colonies and proliferated.

[0043]

(6) Sorting of hybridomas

A culture supernatant of the Fas ligand-expressed COS cells was used as an effector molecule, and a transfectant which expresses a Fas antigen on a cell surface was used as a target to sort out hybridomas in the culture supernatants which blocked the killer activity of the Fas ligand molecule against the transfectant.

[0044]

① Preparation of soluble Fas ligand molecule

A soluble Fas ligand molecule present in the culture supernatant of the Fas ligand-expressed COS cells was used as the Fas ligand molecule. More specifically, after Fas ligand-$PMKIT_{neo}$ was transfected into COS cells by the DEAE-dextran method, the cells were cultured with 100 ml of a 10% FCS-DME medium for a week, followed by collection of a culture supernatant thereof. The supernatant was sterilized through a filter having a pore size of 0.45 μm, thereby providing it as the soluble Fas ligand molecule.

[0045]

② Preparation of target cells

WR19L cells in which a human Fas gene had been transfected were used as the target cells. The transfection of the human Fas gene into WR19L (ATCC TIB52) was performed in accordance with a method known *per se* in the art. More specifically, the cells were prepared by reference to literature by Hanabuchi et al. (Proc. Natl. Acad. Sci. USA, Vol. 91, No. 11, pp. 4930-4934, 1994). The Fas-WR19L cells thus obtained were cultured and adjusted to 2 x 10^5 cells/ml with a 10% FCS·RPMI medium.

[0046]

③ Screening assay

The soluble Fas ligand molecule prepared in the step ① was first diluted to 1/12 with a 10% FCS-DME medium. A 96-well flat-bottomed plate (manufacture by Corning) was used to add 25 μl of the diluted solution and 25 μl of the culture supernatant of the hybridoma to each well, followed by incubation at 37°C for 1 hour. Thereafter, the Fas-WR19L cells prepared in the step ② were added in a proportion of 50 μl/well and incubated for 12 hours under conditions of 37°C and 5% CO_2.

An Alamar Blue™ assay kit (product of Kanto Chemical Co.) was used to determine survival cell rate, thereby selecting hybridomas in wells which inhibited the killer activity of the soluble Fas ligand molecule against the Fas-WR19L cells.

[0047]

(7) Cloning

The antibody-producing cells (hybridomas) were separately poured into wells of a 96-well microplate by the limiting dilution technique so as to give a cell concentration of one cell/well to culture each cell. After culturing for 10 days, the proliferation of a single colony could be confirmed. Therefore, the process of detecting the antibody by the blocking of killer activity was performed again. As a result, clones reacting specifically with the Fas ligand were obtained. An antibody was recovered from a culture supernatant containing the hybridoma of a monoclone, thereby obtaining a monoclonal antibody which specifically reacts with the intended Fas ligand.

The thus-obtained hybridomas which separately produce a monoclonal antibody were named "NOK". Examples thereof may include hybridoma cell lines deposited as Accession Nos. FERM BP-5044 (NOK1), FERM BP-5045 (NOK2), FERM BP-5046 (NOK3), FERM BP-5047 (NOK4) and FERM BP-5048 (NOK5) in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology.

[0048]

(8) Characterization of monoclonal antibody

Characterization ① (staining of FasL-expressed cell)

Whether an antibody produced by the thus-obtained hybridoma, for example, the cell line NOK5, reacts with a

Fas ligand expressed on a cell surface or not was investigated by comparing the Fas ligand-expressed L5178Y cells with the L5178Y cells (ATCC CRL 1723) which are a parent strain thereof.

The L5178Y cells were separately adjusted to 1 x 10^6 cells/ml with PBS. These cells (each, 1 x 10^6 cells) were placed into tubes (Falcon No. 2008). Then, 100 μl of a culture supernatant of Hybridoma NOK5 were placed to conduct a reaction for 30 minutes on a water bath. The reaction mixtures were then centrifugally washed (1500 rpm, 1 minute, twice) with PBS, and 1 μl of FITC-anti-mouse Ig's (product of Cosmo Bio/ Cappel) was added to conduct a further reaction for 20 minutes on an ice bath. After the reaction, the reaction mixture was centrifugally washed twice with PBS and suspended in 200 μl of PBS, followed by determination by means of an FACScan.

[0049]

As a result, it was revealed that the antibody produced by NOK5 reacts with the Fas ligand-expressed L5178Y cells, but does not react with the L5178Y cells of the parent strain thereof as illustrated in FIGs. 1 to 3. Namely, as illustrated in FIGs. 2 and 3, the stain patterns of the parent L5178Y cell strain do not differ from each other irrespective of the addition of the NOK5 antibody. As illustrated in FIG. 1, however, the stain patterns of the Fas ligand-L5178Y cells clearly differ from each other depending on whether the NOK5 antibody is

added or not.

The use of the cell lines of NOK1 to NOK4 achieved the same results as those in the above-described NOK5.

[0050]

Characterization ② (determination of subclass)

Subclasses of monoclonal antibodies produced by the hybridomas NOK1 to NOK5 were determined.

The subclasses were determined by using MAB typing kits (products of PharMingen Co.) in accordance with the accessory protocol. As a result, the subclasses of NOK1, NOK2, NOK3, NOK4 and NOK5 were mouse IgG_1, mouse IgG_{2a}, mouse IgM, mouse IgG_3 and mouse IgG_{2a}, respectively.

[0051]

Characterization ③

As described above, the Alamar Blue™ assay kit (products of Kanto Chemical Co.) was used to determine the survival cell rate, thereby selecting hybridomas in wells which inhibited the killer activity of the soluble Fas ligand molecule against the Fas-WR19L cells. When the measurement was carried out in accordance with the method described above in the step (6) ①-③, the monoclonal antibodies separately produced by the hybridomas NOK1 to NOK5 inhibit the killer activity of the soluble Fas ligand molecule against the Fas-WR19L cells at a high rate of at least 98% as shown in the following Table.

[0052]

[Table 1]

Clone	Survival rate
No addition of any antibody	3.5%
Addition of culture supernatant of NOK1	99.3%
Addition of culture supernatant of NOK2	105.2%
Addition of culture supernatant of NOK3	101.0%
Addition of culture supernatant of NOK4	109.8%
Addition of culture supernatant of NOK5	98.2%

[0053]

[Effects of the Invention]

Since the monoclonal antibodies against Fas ligand according to the present invention specifically react with a Fas ligand, they can serve to elucidate signal transfer mechanism for inducing apoptosis against cells, and a Fas system, for example, by analyzing the interaction between a Fas antigen and its ligand.

The monoclonal antibodies against Fas ligand according to the present invention are useful in immunothearpy and immunodiagnoses, and industrial fields associated with them. More specifically, the monoclonal antibody against Fas ligand is reacted with cells in blood, and a secondary antibody of a fluorescent marker is further bound thereto to measure the conjugate by flow cytometry or a fluorescent microscope, thereby being able to confirm that the Fas ligand has expressed in what cells. The monoclonal antibody against Fas ligand can be easily bound to a fluorochrome such as FITC or PE. Accordingly, analysis can be conducted without using any secondary antibody. Therefore, the monoclonal antibodies according

to the present invention are very useful in fields of diagnoses and fundamental researches.

[0054]

When the monoclonal antibody according to the present invention is reacted with tissues and the like taken out of a patient suffered from various diseases (for example, an autoimmune disease, rheumatism and hepatitis), what tissue Fas ligand-expressed cells exist in can be determined. This permits the diagnoses and treatments of the various diseases. Since the monoclonal antibodies against Fas ligand inhibit the reaction (binding) of a Fas ligand, they are useful in treating diseases such as hepatitis and rheumatism. When an antibody-producing gene is synthesized from the monoclonal antibody according to the present invention, and only a region related to binding with a Fas ligand is transplanted into a human IgG antibody, a humanized antibody can be obtained. The humanized antibody is useful in treating the many diseases described above.

[Brief Description of the Drawings]

[FIG. 1]

FIG. 1 is an FACScan chart illustrating stain patterns of Fas ligand-L5178Y cells.

[FIG. 2]

FIG. 2 is an FACScan chart illustrating a stain pattern of a parent L5178Y strain in the case where no NOK5 antibody was added.

[FIG. 3]

FIG. 3 is an FACScan chart illustrating a stain pattern of the parent L5178Y strain in the case where an NOK5 antibody was added.

[Description of characters]

1: No NOK5 antibody was added

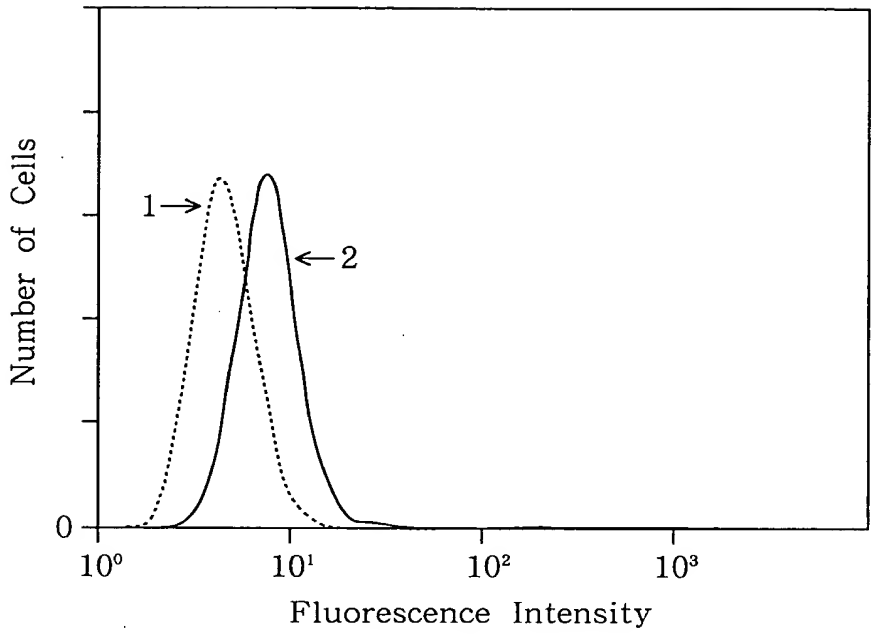
2: An NOK5 antibody was added

3: No NOK5 antibody was added

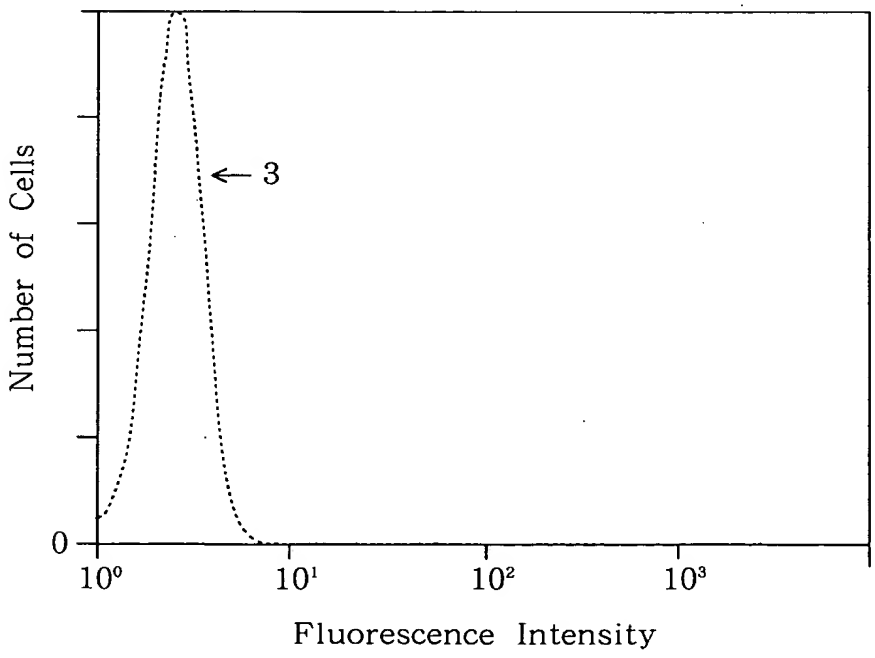
4: An NOK5 antibody was added

[Document Name] DRAWINGS

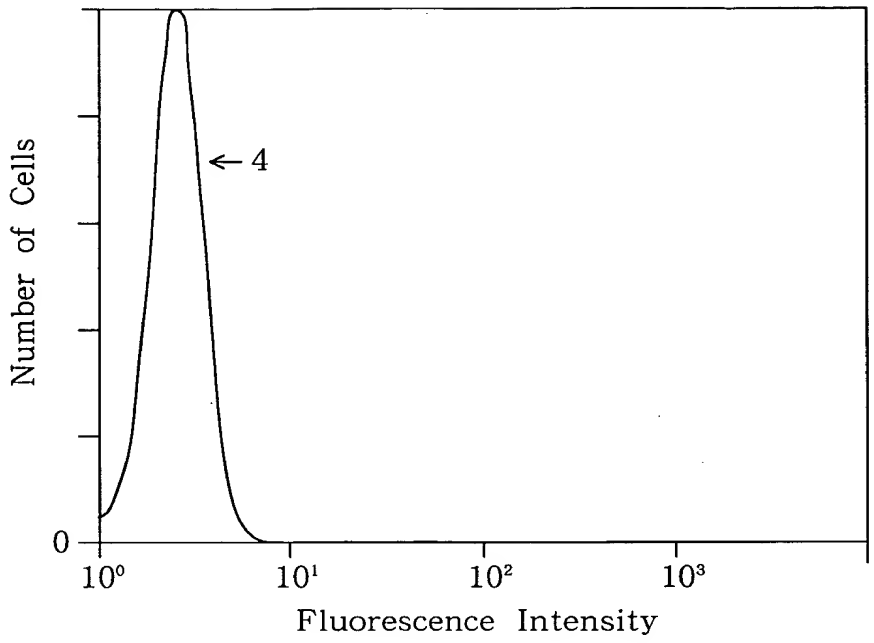
[Figure 1]



[Figure 2]



[Figure 3]



[Document Name] Abstract

[Abstract]

[Object] To provide monoclonal antibodies, which specifically react with a Fas ligand present on a cell surface, active fragments thereof, method for detect a Fas ligand in a solution, a kit for use in detecting the Fas ligand, a production process of the monoclonal antibodies, and hybridomas separately producing the monoclonal antibodies.

[Constitution] A monoclonal antibodies, which specifically react with a Fas ligand or an active fragments thereof. A production process of the monoclonal antibodies which specifically react with a Fas ligand, including the step of immunosensitizing the animals like the mouse with Fas ligand-expressed COS cells. A hybridomas separately producing a monoclonal antibody, which specifically reacts with a Fas ligand. A method of detecting a Fas ligand in a solution (blood, culture supernatant, body fluids, urine), and a kit for use in detecting a Fas ligand.

[Selected Figure of Drawings] None.

INTERNATIONAL FORM

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page.

Depositor:

Name: SUMITOMO ELECTRIC INDUSTRIES, LTD.
Noritaka Kurauchi, Esq.
President

Address: Postal Code: 541
5-33, Kitahama 4-chome, Chuo-ku, Osaka-shi, Osaka

I . Indication of Microorganisms	
(Indication for Identification Given by Depositor) NOK1	(Deposition No.) FERM BP- 5044
II . Scientific Nature and Taxonomic Position	
A document in which the following facts were described has been attached to the microorganisms set forth in Row I. ☒ Scientific Nature ☒ Taxonomic Position	
III . Acceptance and Receipt	
This international depository authority receives the microorganisms set forth in Row I accepted on March 20, 1995 (original depositing date).	
IV . Acceptance of request for transfer of jurisdiction	
This international depository authority accepted the microorganisms set forth in Row I on (original depositing date), and accepted a request for transfer of jurisdiction from the original deposit to a deposit based on Budapest Treaty on .	
V . International depository authority	
Name: National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology Osamu Suzuki, Dr., DIRECTOR GENERAL. Address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, Japan Date: March 20, 1995	

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[Document Name] Data of authoritative correction

[Document Corrected] Application for Patent

<Authorized Information · Additonal Information>

[Applicant for Patent]

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[Name] Shigeaki Nishikawa

[Description of Material Submitted]

[Material Mame Submitted] Receipt in the case of a deposit 2

Information of Applicant's Personal History

Identification Number [000002130]

1. Date Changed August 28, 1990

[Reason for change] New registration

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